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Analysis of Cocaine, Heroin, and Their Metabolites in Saliva

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Methods have been developed to analyze for cocaine, heroin and their metabolites in human saliva and the results compared to urinalysis. Analysis of 26 samples showed that 14 positive by urinalysis and 16 positive by saliva analysis. Thus, saliva testing may be competitive to urine testing for detection of drug use. Only a small fraction of the saliva is necessary for the analysis so that further testing may be employed. Also, the metabolite profiles are shown for further analytical development.

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CONTENTS

Introduction

One problem of particular concern in the Navy drug deterrence program is the question of observation during collection of the urine specimen. Although the guidelines provide for direct observation, some personnel are quite adamant in providing their specimen in private. Direct observation of the urine collection was instituted to prevent dilution or substitution of samples. Anecdotal evidence from personnel in drug rehabilitation clinics suggests that they had defeated the system for many years by switching samples, even under direct observation.

Saliva analysis would be an ideal alternative to urinalysis. Saliva can be obtained under direct observation without the privacy issues involved in urinalysis pertaining. Little problem occurs in obtaining saliva since it is present at all hours when an individual is awake.

Another criticism of urinalysis is that the concentrations of drugs or metabolites present in urine has no correlation to an individual's intoxication.[1] In contrast to this, previous research has indicated that many drugs are present in saliva in concentrations that parallel those found in blood.[2,3,4,5] Thus, there would be a better relationship to the concentrations of drugs in saliva and the state of intoxication of an individual.

Few studies of cocaine in saliva have been completed, with most only relying upon detection by immunoassays.[2,3] The first report of the detection of cocaine in saliva came from a study of the metabolism of radiolabeled cocaine.[6] Later, cocaine was found in the saliva of impaired drivers.[7] Most studies show similar pharmacokinetics of plasma and saliva levels for cocaine. However, in a recent controlled study, cocaine was found 5-10 days after abstinence of chronic addicts.[8] This long detection window was attributed to accumulation in deep body compartments.

Only two studies on the saliva levels of morphine have been reported.[9,10] The latter was a controlled study examined the metabolism of heroin in plasma and saliva. This study followed the metabolism of heroin by several immunoassays. It was detected for only 2-4 hours in each medium but this short detection window may have been due to the insensitivity of the immunoassays employed.

In collaboration with Steve Magura at the Narcotic Drug Research Institute (NDRI) in New York City, we evaluated a commercial collection device called the Saliva-Sac for the collection of saliva. NDRI has a contract from the National Institute on Drug Abuse to collected urine samples from people recently put on parole or probation. Previous studies suggested that over 50% of these people would be positive for cocaine or heroin. This high positive rate allowed for a easier evaluation of saliva as an indication for drug use.

This study had four objectives: (1) Develop sensitive mass spectrometric based techniques to analysis drugs in saliva. 2) Determine what metabolites may be present in saliva and their approximate concentrations. (3) Evaluate the Saliva-Sac as a collection device for saliva. (4) Compare saliva to urine for ease of detection and generation of false negatives. This initial study concentrated on the drugs cocaine and heroin.

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Experimental

For analysis, we developed an LC/MS scheme that requires no extraction step. The saliva sac is washed with running water, patted dry with a disposable paper towel and opened with scissors. The sample is poured into a labeled vial. In these preliminary experiments all samples were removed from this vial when required using disposable pipettes. In later experiments, aliquotes will be poured from the vial for analysis. In this way nothing could contact the sample to cause inadvertent contamination.

For the immunoassays, 50 µl of saliva or urine was analyzed by Syva EMIT-ST using a modified protocol similar to urine testing. A calibration curve was generated with standards to allow approximation of the drug concentration in the saliva and urine. The curve was fitted to the equation log(drug concentration) = slope*(absorbance change-absorbance change at zero)+intercept with the change in absorbance printed by the photometer. This differs from the normal operation of the EMIT-ST instrument in that no quantitation is normally provided. Also, a standard was not analyzed for each specimen. Instead, just the change in absorbance was obtained and the concentration calculated from the calibration curve.

Reliable concentration could only be obtained above 150 ng/ml for both cocaine and opiates using these procedures. Because saliva samples generally had lower concentration of drugs than urine, only a few were tested by immunoassay. A modified immunoassay could be developed to test these lower drug levels.

All urine samples were analyzed by EMIT for cocaine and opiates. The quantity of drug was estimated using the same calibration curve as for saliva. In a few cases, see discussion, the urine was analyzed by LC/MS. Otherwise, no confirmation of the positive was performed.

For LC/MS, 200 µl of either saliva or urine was measured with a disposable Eppendorf pipette into a 1 ml plastic Titertube (Bio-Rad). The internal standard was added (10 µl, containing 50 ng of d3-benzoylecgonine and 50 ng nalorphine). The sample was vortexed and all the solution injected into the HPLC.

The HPLC conditions are given in Table 1.

Table 1 - HPLC Conditions

Column:	Alltech/Applied Science Econosphere C8, 250 x 4.6 mm
Solvent A:	0.1M Ammonium Acetate
Solvent B:	10:90 Solvent A:Acetonitrile
Flow:	1.3 ml/minute, wait 1 minute then: Linear Gradient 0-100%, 10 minutes

The Mass Spectrometer (HP-5970 attached to a Vestec Thermospray unit) and Thermospray system were manually started about 5 minutes after the HPLC analysis had begun. The mass spectrometer was operated in the selected ion mode to detect cocaine, heroin and all their metabolites. The dwell time was 100 ms for each ion scanned.

Results and Discussion

The Navy urinalysis program requires that two independent analysis techniques determine that a specimen is positive before the results are reported as positive. In this program, 100 ml of urine is usually submitted for analysis. For testing, 5 ml of a sample is aliquoted and screened by up to 7 different immunoassays for individual drugs and drug classes. Each immunoassay requires 50-100 μl of sample, the rest is discarded. If positive by an immunoassay, another 20 ml of urine is obtained and extracted, derivatized and analyzed by gas chromatography/mass spectrometry (GC/MS). If positive by GC/MS, the sample is reported positive to the submitting authority and the remaining 35-75 mls of urine are frozen for possible retesting by either the Navy Drug Screening Laboratory or any other certified laboratory that the individual may request. This sequestered sample is an important part of the system as it allows an individual to question the testing results and obtain an independent analysis if desired.

A major problem faced in saliva analysis is the low volume of sample that can be obtained. Saliva samples are rarely over 3 ml in volume with 1 ml being the norm. Since at least 350 μl must be used for the 7 immunoassays, this only leaves 650 μl for a confirmation assay. This small volume makes GC/MS analysis considerably more difficult than in testing of urine.

LC/MS

To conduct this study, we developed an analysis technique for cocaine and heroin and their metabolites based upon Liquid Chromatography/Mass Spectrometry (LC/MS) using only 200 μl of the collected sample. For GC/MS the sample must be extracted into an organic solvent and concentrated to a few microliters. Only 1-5 μl may be used for each analysis and aqueous samples must be avoided. This extraction and concentration step is quite time consuming and often results in large losses of sample. LC/MS has the advantage over GC/MS in allowing the analysis of larger volumes of samples (50-500 μl) and the direct analysis of aqueous solutions. LC analysis has been employed before for the analysis of cocaine and its metabolites in urine.[11]

A typical response for each of the metabolites of cocaine are shown in Figure 1. To obtain these selected ion traces, a solution containing 50 ng of each compound was injected. Traces for an individual with a high level of drugs in their saliva is shown in Figures 2 and 3. The chromatography for cocaine and methylecgonine is poor. Normally, drugs such as cocaine are chromatographed using phosphate buffers. For LC/MS, the conditions available for analysis are limited since the buffers used must be volatile and in high concentration. Also, only acetonitrile/water or methanol/water eluents are practical. The noise present in the m/z 304 trace around the cocaine peak is due to the high acetonitrile concentration needed to elute cocaine. High organic concentrations produce instability in the thermospray process and generate noise. For these preliminary experiments, the poor chromatography and poor spectra for cocaine is acceptable. Later research will focus on improving these results.

In contrast to GC/MS spectra, Thermospray spectra typically only show protonated molecular ions with little or no fragmentation. Thus only, the relative retention time and the intensity of a single ion are available for determination of the presence of a substance. If more information, such as a fragmentation pattern is necessary, tandem mass spectrometric techniques must be used. These will be employed in future experiments for a selected number of specimens.

The Mass Spectrometer and Thermospray system were manually started about 5 minutes after the HPLC analysis had begun. This allowed time for the sugar that was contained in the Saliva-Sac to elute. If this precaution is not observed, the Thermospray system will quickly become plugged. However, because of the variation in start time, the absolute retention time varies between samples. The relative retention time is not affected.

Figure 1 - LC/MS Selected Ion Monitoring for Cocaine

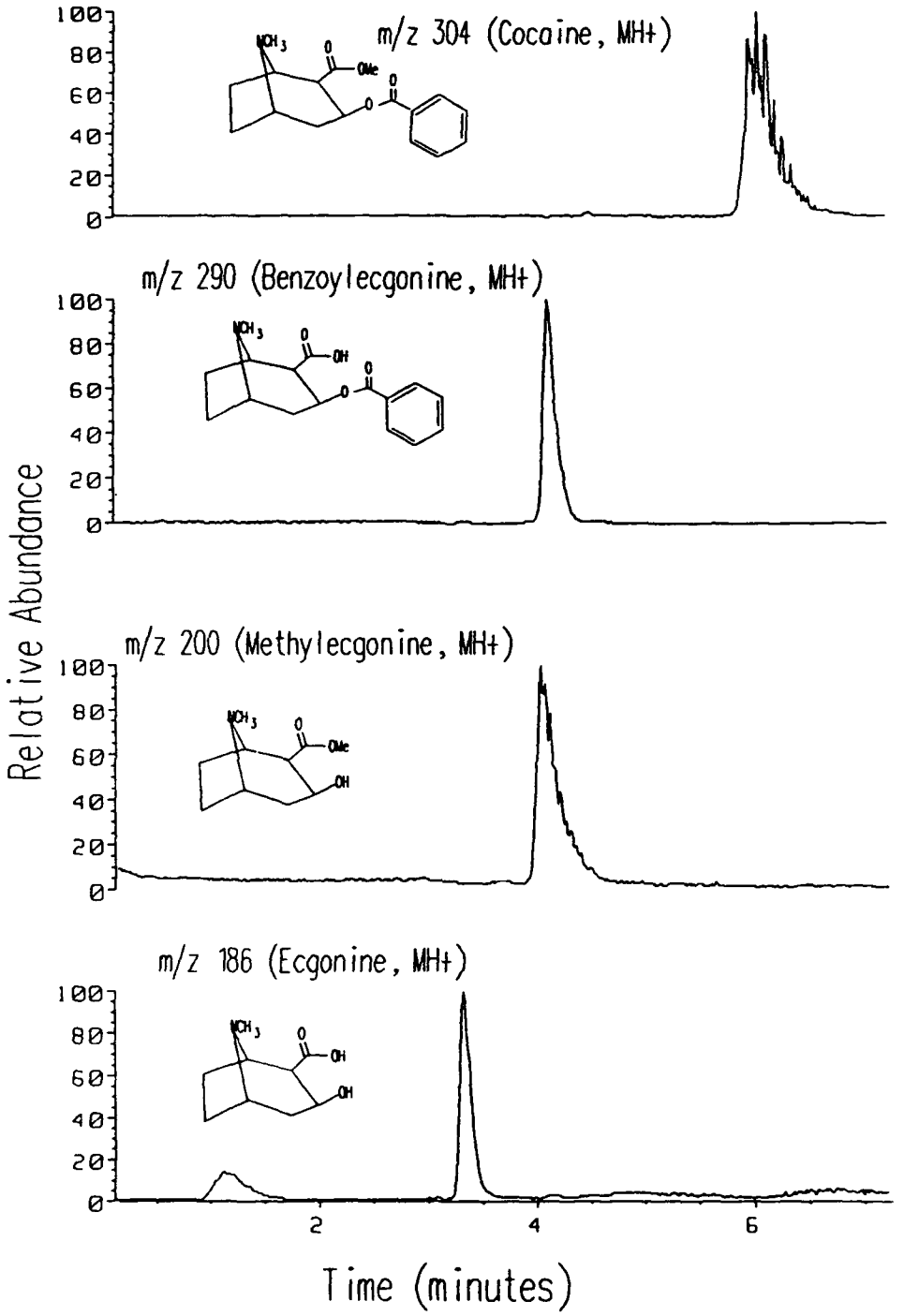


Figure 2 - LC/MS Selected Ion Monitoring Traces of Saliva from a Cocaine User

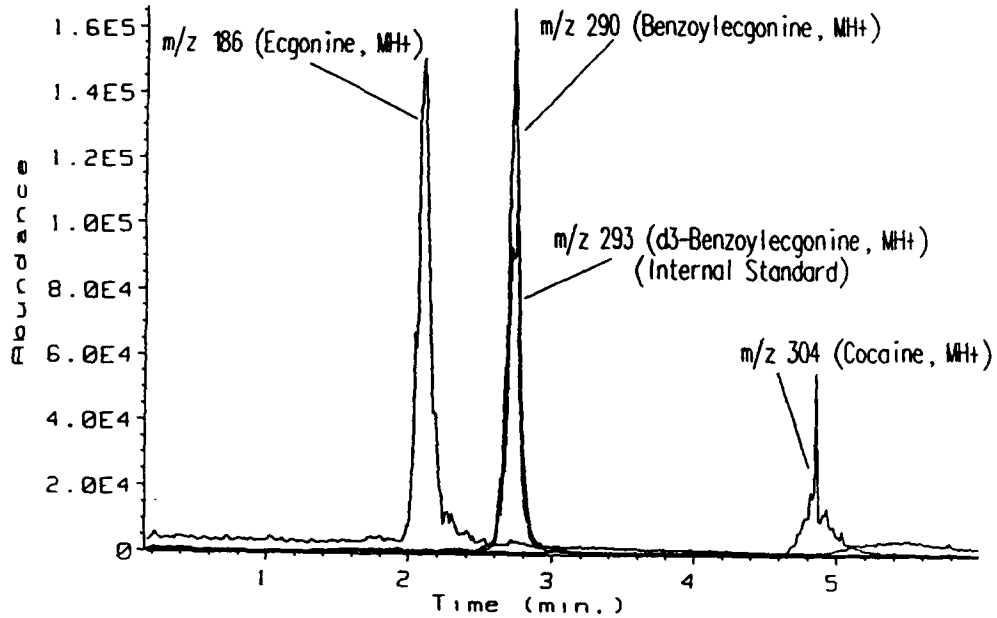
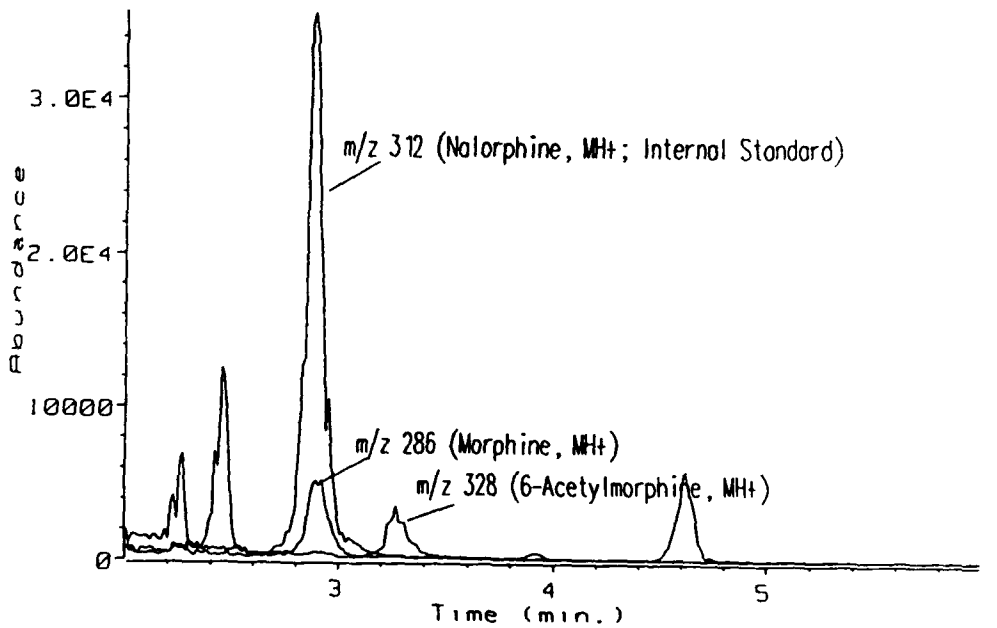


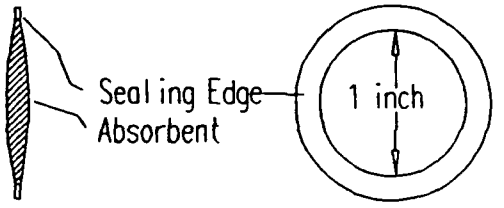
Figure 3 - LC/MS Selected Ion Monitoring Traces of Saliva from a Heroin User



Saliva Collection - The Saliva-Sac

The Saliva-Sac is a "pill" consisting of a bag made of dialysis material and containing an osmotically active material. It is manufactured by BioQuant, Inc., Ann Arbor, MI. A schematic diagram of this device is shown in Figure 4. The dialysis membrane serves two purposes: (1) It filters the aqueous portion of the saliva from the mucopolysaccharides which give saliva its slimy feel. The Saliva-Sac produces a solution much like water. (2) It controls the rate of absorption of water to prevent a dry-mouth feel. The osmotically active material (sucrose in the devices employed in this study) facilitates the flow of water. The Saliva-Sacs used in this study were of the stimulatory type containing citric acid which increases the production of saliva.

Figure 4 - Schematic of the Saliva-Sac



One problem occurs with the Saliva-Sac; the large amount of sucrose used as the osmatic material produces a saturated solution when dissolved by the saliva. This changes the concentration of the saliva and any materials contained inside by diluting it with sugar. This dilution effect was found to be about a factor of two in this study (see Table 2). The manufacturer recommends measuring the density of the saliva solution and adjusting the concentrations of drugs accordingly. No adjustment was made to the data in this study because this factor should be constant and inconsequential if the saliva is always obtained in the same manner. An adjustment to the drug concentrations should be made if they are compared with other collection devices or for correlation to blood plasma levels.

Table 2 - Discrimination of Saliva-Sac for Cocaine at 25°C

	Cocaine (ng/ml)
Initial concentration of soaking solution:	1592
Final concentration of soaking solution:	1314
Concentration in Sac 1:	568
Concentration in Sac 2:	655

Another potential problem with the Saliva-Sacs is the passage of molecules through the dialysis membrane. The rate of diffusion of molecules is related to their charge, size and shape.[12] Drugs of abuse are larger and often positively charged relative to water. Thus their passage should be restricted relative to water and their concentration reduced. This accounts for some of the reduction in concentration of the drugs compared to the media shown in Table 2. However, since all materials would show different behaviors, each should be measured individually to gain accurate quantitation data. These experiments are in progress.

Concentration of Drugs in Saliva and Urine of Drug Users

The data are divided into three overlapping tables. Table 3 lists the amount of drug or its metabolites in urine or saliva. The concentration of the drugs in Table 3 for urine was measured mostly by immunoassay. In a few cases (not shown) the concentrations of drugs were confirmed by LC/MS. The concentration in saliva was the TOTAL of all the various metabolites as measured by LC/MS. In only a few cases were immunoassays run as the sensitivity would have been inadequate. Because of the cross reactivities, EMIT is most sensitive to benzoylecgonine and morphine with the other metabolites being only sightly cross-reactive. Table 4 gives the concentrations of each of the metabolites in saliva for only the positive samples.

Table 3 - Concentration of Total Cocaine Species and Opiates in Urine and Saliva (ng/ml)

Sample	Cocaine(Urine)	Cocaine(saliva)	Opiates(urine)	Opiates(saliva)
** 1	(627)	14	-	-
26	-	-	-	-
** 45	4470	-	-	-
52	1970	315	-	-
91	-	163	-	-
115	1400	42	-	-
** 123	(511)	232	-	-
130	-	-	-	-
136	-	-	-	-
140	-	-	-	-
149	150	17	3000	3
151	3700	78	1288	?
159	9200	34	-	-
171	-	-	-	-
175	14600	478	1600	25
184	-	-	-	-
185	5900	56	-	-
194	4100	81	639	-
** 195	(571)	17	-	-
212	7900	6	325	?
216	2381	22	-	-
222	-	-	-	-
** 223	(600)	17	-	194
** 233	181	857	1627	69
272	11000	67	-	-
"DF"	236	64	-	-

Key: ? = Possible low level
** = Sample discussed in text
(xxx) Values by LC/MS, negative by immunoassay

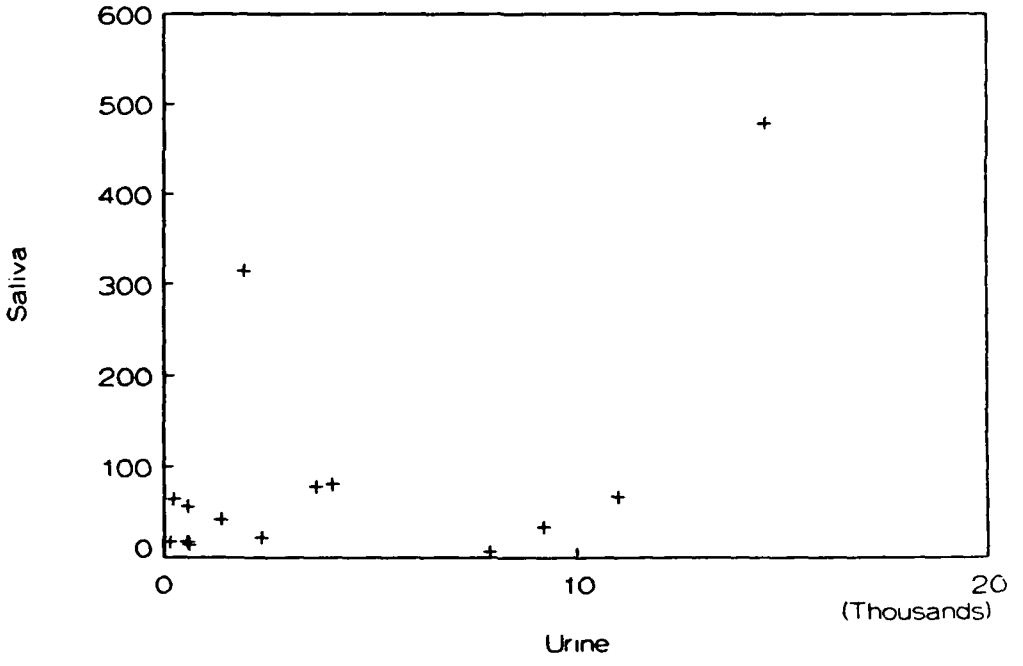
Table 4 - Concentration of Cocaine and Heroin Metabolites in Saliva Positives (ng/ml)

Sample	Ecg.	MEcg.	BE.	Coc.	Mor.	MAM	Her.
1	-	-	10	4	-		-
52	87	56	166	6	-	-	-
91	20	-	122	21	-	-	-
115	-	-	-	42	-	-	-
** 123	232	-	-	-	-	-	-
149	-	-	13	4	3	?	-
151	-	-	-	65	13	?	-
159	25	-	9	-	-	-	-
175	-	109	137	232	25	-	-
185	-	22	13	21	-	-	-
194	-	-	65	16	-	-	-
195	6	-	11	-	-	-	-
212	1.3	-	2.3	2.5	-	?	-
216	11		4	7	-	-	-
223	-	-	4	13	43	103	48
233	391	-	356	110	42	27	-
272	-	18	41	8	-	-	-
"DF"	-	-	37	27	-	-	-

Key:
Ecg. = Ecgonine
Mecg. = Methylecgonine
BE. = Benzoylecgonine
Coc. = Cocaine
Mor. = Morphine
MAM = 6-Acetylmorphine
Her. = Diacetylmorphine (Heroin)
** = Sample probably in error - see text

A scatter plot showing the correlation of drug concentrations in urine and saliva is shown in Figure 5. The one point from sample 223 was removed from this plot to allow the lower concentration sample to be shown. Linear regression on these data points gives a slope of 0.015 with a correlation coefficient of 0.72.

Figure 5 - Correlation of Cocaine in Saliva and Urine



Several samples deserve comment. In five out of 26 samples, there were drugs present in the saliva yet the urine was negative by immunoassay. High salt concentrations or abnormal pH are known to generate false negatives in EMIT, especially when the d.a.u. reagents are being used.[13] For the ST reagents employed in this study, salt or pH do not affect the results. Nevertheless, all samples were tested for chloride concentration and pH to detect possible adulteration. The results are listed in Table 5 and scatter plots of the data are shown in Figure 6 and Figure 7. The pH of all samples was within normal ranges expected for urine. However, the chloride concentrations for many of the samples were above that expected for normal urine which is about 0.14 M. Whether this is attributable to abnormal metabolism in drug users or adulteration of urine is unknown. Also, one urine sample had very low salt concentrations, suggesting dilution of the sample, probably by consuming large amounts of fluids. Significantly, the specimen that had the highest salt concentration was negative by both immunoassay and LC/MS. The saliva from this individual was also negative.

Table 5 - pH and Chloride Concentration of Urine Samples

Sample	pH	[Chloride]
1	5.7	0.36
26	6.2	0.1
45	6.6	0.3
52	5.7	0.23
91	7.0	0.25
115	5.5	0.28
123	7.3	0.14
130	8.6	0.45
136	6.5	0.22
140	5.6	0.36
149	6.8	0.1
151	5.6	0.11
159	5.5	0.28
171	6.7	0.28
184	5.1	0.1
185	6.2	0.043
194	6.6	0.12
195	5.3	0.18
212	9.1	0.076
216	7.1	0.32
222	6.5	0.18
223	7.8	0.33
233	8.2	0.22
272	7.5	0.25
"DF"	5.5	0.25

Figure 6 - Chloride Concentration of Urine

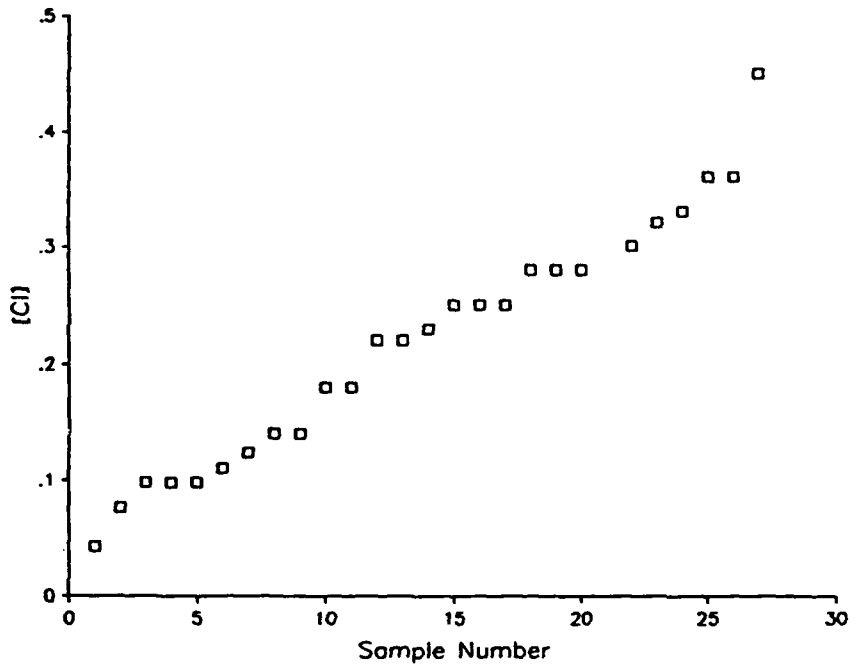
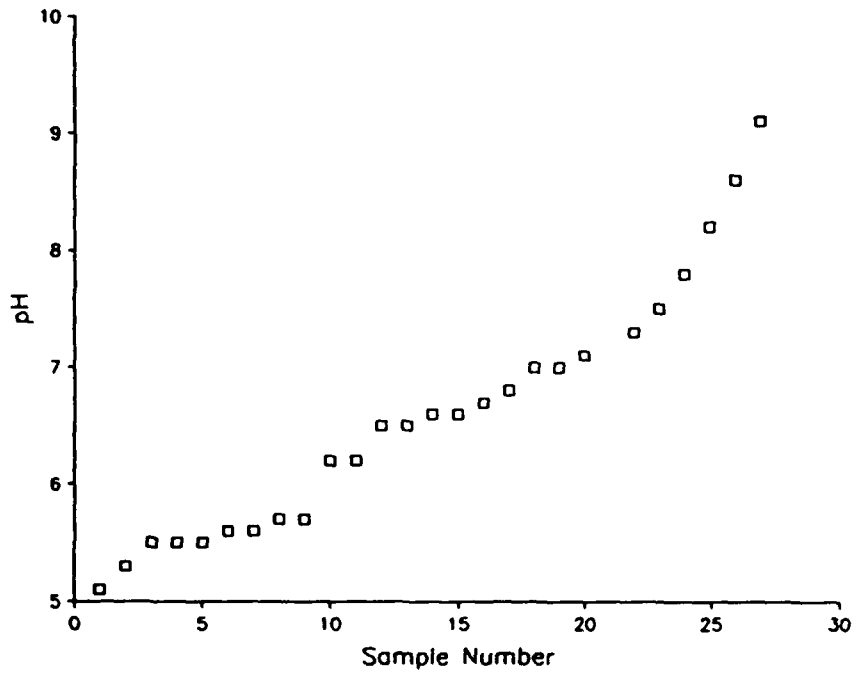


Figure 7 - pH of Urine Samples



The urine of all five specimens where the saliva was positive and the urine was negative were tested by LC/MS and found positive for the corresponding drug classes in saliva. The concentrations of the various drugs are shown in Table 6. Note that in a few cases only ecgonine was present in appreciable quantities. This cross-reacts poorly in the immunoassay so would not generate a positive. Ecgonine is both a metabolite of cocaine and a degradation product. Why the normal metabolite (benzoylecgonine) is not present is unknown.

Table 6 - Drug Metabolite Concentrations for Selected Urine Specimens

Sample	Ecg.	MEcg.	BE.	Coc.	Mor.	MAM	Her.
1	592	-	35	-	-	-	-
45	-	-	7300	-	-	-	-
123	511	-	-	-	-	-	-
195	571	-	?	-	-	-	-
[illegible]	600	-	-	-	105	134?	-
233	131	31	66	-	539	130?	-

Samples 45 and 123 are listed in Table 6 because they show unusual patterns. Sample 45 had very high levels of benzoylecgonine in the urine, but no other metabolites. Also, the saliva sample had only trace amounts of benzoylecgonine. The results on saliva sample 123 may be in error. It was yellow when received and smelled of urine. Discussions with the collection personnel suggest that this sample may have been inadvertently mixed with urine. Both the urine and this sample have similar drug profiles in that only ecgonine is present.

Conclusions

A rapid screening and confirmation of cocaine and opiate use through saliva analysis has been developed. Only a small fraction of the saliva is necessary for the analysis so that further testing may be employed. Detection of cocaine and opiate use through testing of saliva is a practicable alternative to urinalysis. Of the 14 samples tested positive by urine immunoassay, all but one were positive by saliva analysis. In addition, 3 samples that were negative by immunoassay either through adulteration, or low concentrations of the appropriate metabolites were positive by saliva analysis. Ecgonine and benzoylecgonine were present in most of the samples. However, the presence of all metabolites should be screened. Future work will concentrate on the testing of more samples and improvements in the testing methodology.

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